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Comparative studies on the cumene hydroperoxide- and NADPH-supported *N*-oxidation of 4-chloroaniline by cytochrome *P*-450

Peter HLAVICA, Ines GOLLY and Jutta MIETASCHK
Institut für Pharmakologie und Toxikologie der Universität, Nussbaumstrasse 26, D-8000 München 2, Federal Republic of Germany

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1. The present study confirms that cytochrome *P*-450 can act as a catalyst in the cumene hydroperoxide-supported *N*-oxidation of 4-chloroaniline. 2. Analogous to the NADPH/O_2-driven *N*-oxidation process, product dissociation is likely to limit the overall rate of cytochrome *P*-450 cycling also in the peroxidatic pathway. 3. The oxy complexes involved in either metabolic route differ with respect to stability, spectral properties and need for thiolate-mediated resonance stabilization. 4. With the organic hydroperoxide, the metabolic profile is shifted from the preponderant production of *N*-(4-chlorophenyl)hydroxylamine to the formation of 1-chloro-4-nitrobenzene. This finding suggests that the peroxide-sustained *N*-oxidation mechanism differs in several ways from that functional in the NADPH/O_2-dependent oxenoid reaction. Thus one-electron oxidation, triggered by homolytic cleavage of the oxygen donor, is proposed as the mechanism of peroxidatic transformation of 4-chloroaniline.

Cytochrome *P*-450 (EC 1.14.14.1) has been demonstrated to function as a mono-oxygenase requiring NADPH, molecular oxygen and the presence of substrate to be oxidatively metabolized (White & Coon, 1980). Formation, in the cyclic process, of a discrete active oxygen species has been inferred from spectral studies (Guengerich *et al.*, 1976); such a species has been attributed the character of an oxenoid (Lichtenberger *et al.*, 1976). More recently, it has been shown that cytochrome *P*-450 can act as a peroxygenase too, using a variety of organic and inorganic agents, such as hydroperoxides, peroxy acids, iodosylbenzene, $NaIO_4$ and $NaClO_2$, as co-substrates (O'Brien, 1978). The oxy complex generated from the haemoprotein and one of these oxidants has been proposed to be analogous to that produced in the presence of NADPH and O_2 (Lichtenberger & Ullrich, 1977). Therefore, organic peroxide-dependent drug oxidations mediated by cytochrome *P*-450 have been proposed as suitable models for the NADPH/O_2-supported oxygen activation mechanism operative in the haemoprotein system. The present paper provides evidence that, despite the existence of certain common features, there are pronounced differences between these two types of reaction, with respect to the *N*-oxidation of 4-chloroaniline catalysed by liver microsomal fractions from phenobarbital-treated rabbits.

Materials and methods

Chemicals

Chemicals used in the present study were obtained from the following sources: $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and catalase (EC 1.11.1.6) from Boehringer (Mannheim, Germany); superoxide dismutase (EC 1.15.1.1) from Sigma Chemie (München, Germany); 4-chloroaniline, 1-chloro-4-nitrosobenzene and 1-chloro-4-nitrobenzene from Fluka A.G. (Buchs, Switzerland). *N*-(4-Chlorophenyl)hydroxylamine was synthesized by the method of Bamberger (1890) and stored at −20°C under N_2. Cumene hydroperoxide was purchased from Merck A.G. (Darmstadt, Germany) and purified by established procedures (Armstrong *et al.*, 1950).

Preparation of microsomal fraction

Microsomal fractions from the livers of adult male and female chinchilla rabbits, pretreated with phenobarbital (50 mg/kg body wt.) for 7 consecutive days, were prepared by the method of Jagow *et al.* (1965) and stored at +4°C overnight.

Purification of cytochrome P-450 and NADPH-cytochrome c (P-450) reductase

Partially and highly purified liver microsomal cytochrome *P*-450 from phenobarbital-treated rabbits and detergent-solubilized rabbit liver microsomal NADPH-cytochrome *c* (*P*-450) reductase (EC 1.6.2.4) were prepared as described previously (Autor *et al.*, 1973; Hlavica & Hülsmann, 1979).

Enzyme determinations

The standard assay media for measuring NADPH-dependent *N*-oxidation of 4-chloroaniline contained: 0.15 M-KH_2PO_4/Na_2HPO_4, pH 7.4; 1.2 mM-$NADP^+$; 10 mM-glucose 6-phosphate; 6 mM-$MgCl_2$; 5 μg of glucose 6-phosphate dehydrogenase/ml of mixture; 1 mM-4-chloroaniline, and liver microsomal protein from phenobarbital-treated rabbits equivalent to 0.2 μM-cytochrome *P*-450. Reactions were carried out for 3 min at 37°C.

The reaction mixtures for measuring cumene hydroperoxide-supported *N*-oxidation of 4-chloroaniline contained: 0.15 M-KH_2PO_4/Na_2HPO_4, pH 7.4; 10 mM-cumene hydroperoxide; 10 mM-4-chloroaniline, and liver microsomal protein from phenobarbital-treated rabbits equivalent to 4 μM-cytochrome *P*-450. The samples were incubated for 7 min at 37°C.

Measurement of difference spectra

Liver microsomal fractions from phenobarbital-treated rabbits were diluted with 0.15 M-KH_2PO_4/Na_2HPO_4, pH 7.4, to a final concentration of 4 μM-cytochrome *P*-450 and divided between two cuvettes. 4-Chloroaniline and/or cumene hydroperoxide were incorporated into the assay media as described in the appropriate legends to the Figures. Alternatively, NADPH was added as indicated. Difference spectra were recorded with a Cary 219 spectrophotometer at 25°C by repetitive scanning; optical path length was 1 cm.

Stopped-flow measurements

Experiments were carried out with an Aminco Dasar/DW-2 spectrophotometer system equipped with an Aminco–Morrow stopped-flow apparatus. Dead time of the instrument was 4 ms. The temperature of the syringes and the mixing chamber was maintained at 25°C.

Rates of interaction of 4-chloroaniline and cumene hydroperoxide with partially solubilized cytochrome *P*-450 were determined by monitoring the increase in absorbance at 424 and 440 nm respectively, after mixing of two solutions, one of which contained 8 μM-cytochrome *P*-450 in 0.15 M-KH_2PO_4/Na_2HPO_4, pH 7.4; the other one contained a solution of either 2 mM- or 20 mM-4-chloroaniline or 20 mM-cumene hydroperoxide in the same buffer. The change in absorbance at 437 nm was recorded, when the reaction of the peroxide with cytochrome *P*-450 was studied in the presence of amine substrate.

The haemoprotein/peroxide-dependent disposal of 1-chloro-4-nitrosobenzene was measured by monitoring the decrease in absorbance at 320 nm after mixing of two solutions, one of which contained 8 μM-cytochrome *P*-450 and the nitroso compound at 0.6 mM in 0.15 M-phosphate, pH 7.4; the other one contained 20 mM-hydroperoxide in the same buffer.

The rate of cytochrome *P*-450 reduction was determined as described previously (Peterson *et al.*, 1978). The final concentrations of the reactants in the observation cell were as follows: 4 μM-cytochrome *P*-450; 50 units of NADPH-cytochrome *c* (*P*-450) reductase/ml of mixture; 0.6 mM-NADPH and 1 mM-4-chloroaniline in 0.15 M-phosphate, pH 7.4.

Pseudo-first-order rate constants for the individual processes were computed from $k = 0.693/t_{0.5}$, where $t_{0.5}$ represents the half-time of the reaction.

Quantification and identification of N-oxidized products

N-(4-Chlorophenyl)hydroxylamine was quantified after conversion into the corresponding nitroso derivative by treatment with $K_3Fe(CN)_6$ (Herr & Kiese, 1959).

1-Chloro-4-nitrobenzene was measured after extraction into tetrachloromethane, t.l.c., with the solvent system hexane/toluene (2:1, v/v), of the extracted material on t.l.c. plates (20 cm × 20 cm) pre-coated with silica gel 60 F_{254} (Merck A.G.) and elution of the separated compound from the gel with propan-2-ol; the absorbance of the eluate was read at 269 nm and compared with a standard curve.

Final confirmation of 1-chloro-4-nitrobenzene as being the metabolic product was achieved by conducting large-scale incubations (100 ml). The combined propan-2-ol eluates of a series of analogous experiments were subjected to reverse-phase h.p.l.c. on a μ-Bondapak C_{18} column (30 cm × 0.4 cm) attached to a Waters model 244 liquid chromatograph. The solvent system was methanol/water (3:2, v/v); the flow rate was 2 ml/min. The eluted sample was introduced via the direct-insertion probe into a CH 7-A Varian mass spectrometer operated at an electron energy of 70 eV; authentic 1-chloro-4-nitrobenzene was analysed as well, for comparison.

Other analytical procedures

Cytochrome *P*-450 was measured as described by Omura & Sato (1964). Protein was determined by the method of Gornall *et al.* (1949) as modified by Szarkowska & Klingenberg (1963).

Results

Nature of N-oxidized products generated during NADPH- and cumene hydroperoxide-dependent microsomal N-oxidation of 4-chloroaniline

Analysis of the incubation mixtures with and without prior $K_3Fe(CN)_6$ treatment reveals that *N*-(4-chlorophenyl)hydroxylamine is the labile primary reaction product arising from NADPH-supported rabbit liver microsomal *N*-oxidation of 4-chloroaniline; when exposed to atmospheric oxygen, the hydroxylamine slowly autoxidizes to yield 1-chloro-4-nitrosobenzene. There is no measurable formation, during the short period of incubation, of nitro- or bisazoxy-arene in the assay media.

On the other hand, a new metabolite was recognized to result from cumene hydroperoxide-dependent microsomal *N*-oxidation of the arylamine. The u.v. spectrum of this new compound exhibits an absorption band at 269nm; analysis of the metabolite by t.l.c. and high-pressure liquid chromatography gives an R_F value of 0.64 and a retention time of 5.8min. Mass-spectral analysis reveals fragments at *m/z* 157(84), 141(4), 127(28), 111(80), 99(26), 85(10), 75(100), 63(6) and 50(41%), the values in the parentheses indicating peak height of the individual fragments relative to that of the ion at *m/z* 75. By comparison of these data with those for authentic material, the unknown product was unequivocally identified as 1-chloro-4-nitrobenzene. The nitro compound constitutes 96% of the total amount of *N*-oxidized products formed, 1-chloro-4-nitrosobenzene accounting for the rest. Since conversion of 4-chloroaniline into the nitro derivative is a six-electron oxidation, we predicted that the corresponding hydroxylamine and nitroso analogues were intermediates in this catalytic process. Indeed, 74 and 91% respectively of the *N*-oxy compounds added were transformed into 1-chloro-4-nitrobenzene, when either 1-chloro-4-nitrosobenzene or *N*-(4-chlorophenyl)hydroxylamine (70μM each) were incubated in standard assay mixtures containing peroxide. As shown in Fig. 1, 1-chloro-4-nitrosobenzene is rapidly disposed of, with $k = 0.15\,s^{-1}$. It is noteworthy that production of 1-chloro-4-nitrobenzene from the nitroso compound is severely blocked in the absence of either microsomal fraction or hydroperoxide. These findings suggest that formation of the nitro analogue is closely associated with the action of an oxygenating species generated through enzymic activation of the organic peroxide.

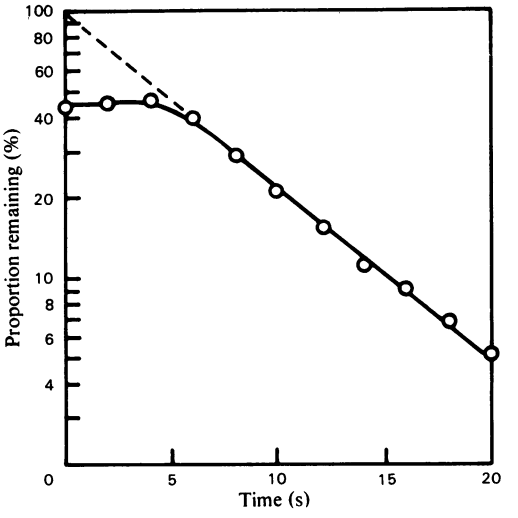

Fig. 1. *Disposal of 4-chloronitrosobenzene in assay media containing cytochrome P-450 and cumene hydroperoxide*

Disposal of the nitrosoarene was measured as described in the Materials and methods section. The final concentrations, in the observation cell, of the reactants were as follows: 0.3mM-1-chloro-4-nitrosobenzene, 10mM-cumene hydroperoxide and 4μM-cytochrome *P*-450 (partially solubilized) in 0.15M-phosphate, pH7.4.

Table 1. *Effect of various modifiers on the cumene hydroperoxide-supported liver microsomal N-oxidation of 4-chloroaniline*

The assay media contained 10mM-4-chloroaniline, 10mM-cumene hydroperoxide, liver microsomal fraction from phenobarbital-treated rabbits equivalent to 4μM-cytochrome *P*-450, and the appropriate agent in 0.15M-phosphate, pH7.4. Incubation time was 7min at 37°C. Results are means of four to five experiments.

Modifier	*N*-Oxygenating activity (% of control)
Boiling (5min at 90°C)	6
N_2 (approx. 99%)	100
CO (approx. 99%)	102
Metyrapone (20mM)	75
Octylamine (10mM)	107
Cyanide (5mM)	29
Azide (5mM)	63
p-Chloromercuribenzoate (5mM)	155
Deoxycholate (1mM)	138
Acetone (15%, v/v)	47
Mannitol (100mM)	114
Superoxide dismutase (70μg/ml)	82
Catalase (140μg/ml)	94

Effect of modifiers on the microsomal N-oxidation of 4-chloroaniline

Table 1 summarizes the effect of various agents on the cumene hydroperoxide-supported microsomal *N*-oxidation of 4-chloroaniline. As expected, heat-inactivated microsomal preparations fail to mediate formation of *N*-oxidized products. Incubation of the assay mixtures under an atmosphere of CO does

not affect *N*-oxidation of the arylamine; this finding suggests that ferrous haemoprotein is not involved in this reaction. There is, however, a marked inhibitory effect of cyanide on the *N*-oxidation process; azide produces a somewhat less pronounced inhibition. Since the two haemoproteins, cytochromes *P*-450 and b_5, account for virtually all the protohaem in the rabbit liver microsomal fraction (Omura & Sato, 1964), but only cytochrome *P*-450 combines with cyanide (Schenkman *et al.*, 1967), this suggests a predominant role of cytochrome *P*-450 in the peroxidatic *N*-oxidation of the amine substrate. Most interestingly, transformation by treatment with deoxycholate or *p*-chloromercuribenzoate of cytochrome *P*-450 to the *P*-420 form significantly stimulates the peroxide-sustained *N*-oxidation reaction. The presence in the reaction mixtures of catalase, superoxide dismutase or mannitol does not appreciably alter the rate of amine oxidation. Hence, H_2O_2, $O_2^{-\cdot}$ or $OH^{\cdot}$ radicals, free in solution, appear not to be involved in the catalytic process.

The effect of pretreatment of rabbits with phenobarbital on both the peroxygenation and the monooxygenation reaction is shown in Table 2. The inducer strongly increases the rate of NADPH-dependent *N*-oxidation of 4-chloroaniline as well as the efficiency of coupling, but only slightly elevates the level of the hydroperoxide-supported *N*-oxidase activity.

Spectral changes generated during NADPH- and cumene hydroperoxide-dependent microsomal N-oxidation of 4-chloroaniline

Addition of NADPH to an aerobic rabbit liver microsomal suspension containing 4-chloroaniline results in rapid reduction of cytochrome b_5, as evidenced by the appearance of an absorbance band at 425nm (Fig. 2*a*). Moreover, a second absorption peak at 452nm forms shortly after incorporation of the reductant. The magnitude of this spectral perturbation increases with time, reaching a maximum at about 7–9min after initiation of the reaction. The 452nm absorbing complex is unlikely to originate from binding to the ferrous haemoprotein of endogenously generated CO, since it is destroyed by the presence of $Na_2S_2O_4$ (results not shown).

The spectral properties of the 452 nm-absorbing species, as evaluated under steady-state conditions,

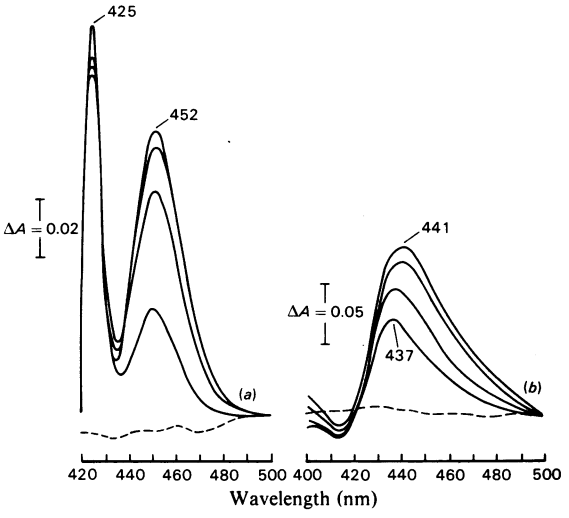

Fig. 2. *Spectral changes associated with the NADPH- and cumene hydroperoxide-sustained microsomal metabolism of 4-chloroaniline*

Standard liver microsomal suspensions, as described in the Materials and methods section, supplemented with 4-chloroaniline (final concentration 0.05 and 1mM respectively), were equally divided between two cuvettes. NADPH (final concentration 1mM) was added to the contents of the sample cell, and scans were taken from 0.5 to 9min after initiation of the reaction (*a*). Alternatively, cumene hydroperoxide (final concentration 1mM) was incorporated into the contents of the experimental cuvette, and scans were taken from 0.3 to 20min after the addition of the oxidant (*b*).

Table 2. *Influence of pretreatment of rabbits with phenobarbital on the cumene hydroperoxide- and NADPH-sustained microsomal N-oxidation of 4-chloroaniline*

Standard incubation media, as described in the Materials and methods section, containing either control or phenobarbital-induced liver microsomal fraction were used for measuring the capacities for *N*-oxidation of 4-chloroaniline; rates of NADPH oxidation were assessed by monitoring the decrease in absorbance at 340nm in reaction mixtures containing 0.1mM-NADPH, 1mM-4-chloroaniline, and limiting amounts of liver microsomal fraction in 0.15M-phosphate, pH 7.4. Results are means ± S.E.M. of three determinations.

	Hydroperoxide-dependent system	NADPH-dependent system		
Pretreatment	*N*-Oxidation (nmol/min per nmol of cytochrome *P*-450)	*N*-Oxidation (nmol/min per nmol of cytochrome *P*-450)	NADPH oxidation (nmol/min per nmol of cytochrome *P*-450)	Coupling efficiency (%)
None	1.14 ± 0.09	0.74 ± 0.06	3.36 ± 0.03	22
Phenobarbital	2.10 ± 0.16	8.38 ± 0.81	7.81 ± 0.18	107

are at variance with those reported for oxyferrous cytochrome *P*-450 (Estabrook *et al.*, 1971). We therefore investigated the spectral changes generated under rapid reaction conditions, using highly purified cytochrome *P*-450 to avoid spectral contributions by cytochrome b_5. When the kinetics of interaction, in the presence of amine, of oxygen with ferrous haemoprotein were studied as a function of wavelength, the typical absolute spectrum of oxycytochrome *P*-450 (Ishimura *et al.*, 1971) with a Soret maximum at 417nm emerged (Fig. 3).

Addition to rabbit liver microsomal preparations of cumene hydroperoxide elicits a short-lived optical change characterized by an absorption in the difference spectrum around 440nm (results not shown). In the presence of 4-chloroaniline, a 437nm-absorbing intermediate is produced immediately after incorporation of the oxidant into the contents of the sample cell (Fig. 2*b*); the absolute spectrum of this complex exhibits an absorption maximum at 420nm (Fig. 3). The initial 437nm-absorbing species undergoes time-dependent transformation, as evidenced by an increase in the magnitude and a bathochromic shift of the absorption peak (Fig. 2*b*). That binding to the pigment of *N*-oxidized metabolites accounts for this spectral alteration seems unlikely: 1-chloro-4-nitrosobenzene interacts with the ferric haemoprotein to yield a difference spectrum with an absorption maximum at 451nm and a minimum at 411nm and 1-chloro-4-nitrobenzene produces a spectral change characterized by a broad absorption peak centred at 452nm and a trough at 422nm (results not shown).

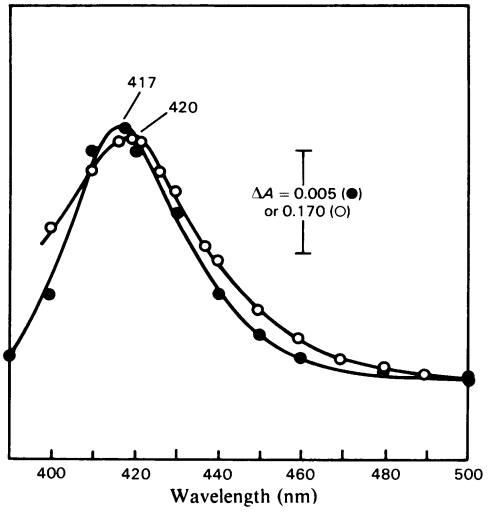

Fig. 3. *Absolute spectra of oxygenated cytochrome P-450, as generated in the presence of NADPH/O_2 or cumene hydroperoxide*

The absolute spectrum of oxyferrous cytochrome *P*-450 (●) was constructed by plotting the rapid reaction kinetics of association of O_2 with highly purified ferrous haemoprotein as a function of wavelength. The final concentrations of the components were as follows: 1mM-NADPH; 250units of NADPH-cytochrome *c* (*P*-450) reductase/ml of mixture; 1mM-4-chloroaniline, and 4μM-cytochrome *P*-450 in an aerobic solution of 0.15M-phosphate, pH7.4. The absolute spectrum of the oxy complex produced from ferric cytochrome *P*-450 and cumene hydroperoxide in the presence of 4-chloroaniline (○) was computed from the initial steady-state difference spectrum presented in Fig. 2(*b*) and the absolute spectrum of the haemoprotein–arylamine adduct, as measured in reaction mixtures containing 1mM-4-chloroaniline and 4μM-cytochrome *P*-450 (highly purified) in 0.15M-phosphate, pH7.4.

Effect of substrate concentration on the patterns of initial velocity of N-oxidation

The results obtained when the final concentration of 4-chloroaniline was varied at two fixed levels of cumene hydroperoxide are shown in Fig. 4(*a*). The double-reciprocal plot of initial rate of *N*-oxidation versus amine concentration yields straight lines having a common point of intersection to the left of the ordinate and above the abscissa. Such kinetic behaviour is consistent with the assumption of the involvement of an ordered bireactant system (Segel, 1975). At a concentration of the oxygen donor of 10mM, which corresponds to about twice its apparent K_m (Fig. 4*b*), the Michaelis–Menten constant for 4-chloroaniline was calculated as 20mM; the $V_{max.}$ has the value of 9nmol of 1-chloro-4-nitrobenzene formed/min per nmol of cytochrome *P*-450. The low affinity for the peroxygenase of the arylamine is likely to originate, in part, from competition between the amine and the hydroperoxide for the same binding site on the enzyme (Fig. 4*a*, inset).

When NADPH and O_2 substitute for the peroxide, the apparent K_m for 4-chloroaniline is 50μM and *N*-oxidation proceeds at an apparent maximum velocity of 8.6nmol of *N*-(4-chlorophenyl)hydroxylamine formed/min per nmol of cytochrome *P*-450 (Fig. 5).

Apparent rates of decomposition of the individual intermediary complexes to release products and ferric haemoprotein were computed from $k = V_{max.}/[E_t]$, assuming that the total amount of cytochrome *P*-450(E_t) present is active in catalysis (Table 3).

Rapid reaction studies

Apparent rates of some of the catalytic steps involved in the metabolic transformation of 4-chloroaniline were assessed by using partially solubilized liver microsomal cytochrome *P*-450 largely depleted of cytochrome b_5. Within each of

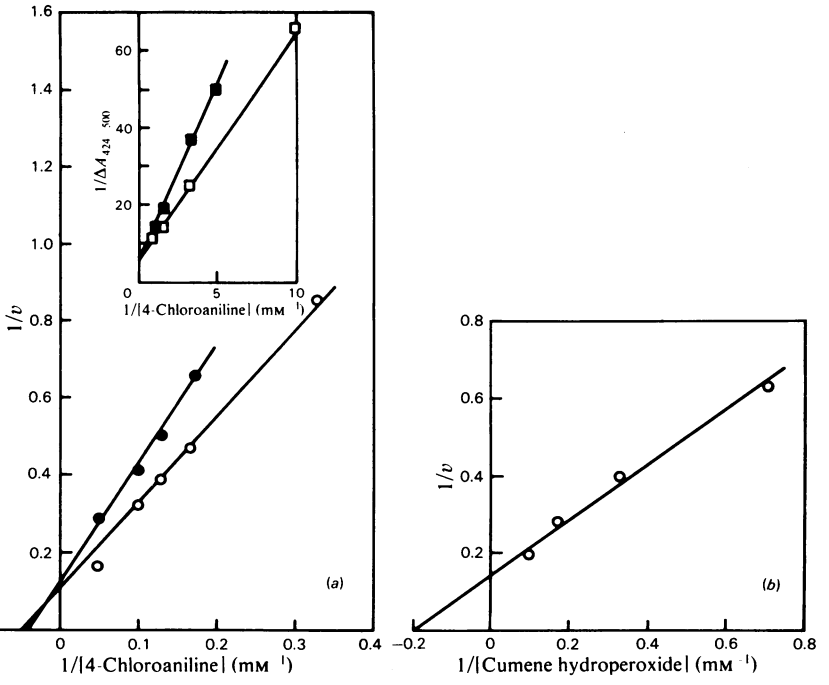

Fig. 4. *Initial-velocity patterns for the cumene hydroperoxide-dependent microsomal N-oxidation of 4-chloroaniline with amine substrate and oxidant as the variables*

(*a*) Microsomal suspensions, as described in the Materials and methods section, containing either 2.5 mM- (●) or 10 mM-cumene hydroperoxide (○) and 4-chloroaniline as indicated were incubated for 7 min at 37°C. The inset of the Figure shows the binding to cytochrome *P*-450 of 4-chloroaniline in the absence (□) and in the presence (■) of 1 mM-cumene hydroperoxide, as measured by monitoring the absorbance change at 424 nm after mixing of the reactants. In (*b*), the assay media contained a fixed level of 10 mM-chloroaniline and the concentrations of cumene hydroperoxide indicated. The initial velocities of *N*-oxidation (v) are expressed in nmol of 1-chloro-4-nitrobenzene formed/min per nmol of cytochrome *P*-450. The experimental points are means of three to five determinations.

the pathways studied, the conditions chosen permit direct comparison of the kinetic data derived from the normalized recordings.

The interaction with cytochrome *P*-450 of 4-chloroaniline, as measured at an amine concentration of 1 and 10 mM respectively, exhibits non-exponential characteristics (Fig. 6*a*). At the two levels of amine substrate tested, almost identical apparent first-order rate constants were obtained. This means that, under both conditions, a steady-state complex is formed.

When cytochrome *P*-450 is mixed with a saturating amount of cumene hydroperoxide to yield the 440 nm-absorbing species, the time course of binding of the oxygen donor is monophasic (Fig. 6*b*), but becomes decidely non-exponential when the peroxide is allowed to interact with the pigment in the presence of 4-chloroaniline (Fig. 6*c*). It is noteworthy that the addition of the amine substrate accelerates formation of the oxy complex.

Experiments on the rate of reduction of the ferricytochrome *P*-450–arylamine adduct were carried out at saturating concentration of NADPH ($K_m = 5\,\mu$M; Dignam & Strobel, 1977) and a molar ratio of NADPH-cytochrome *c* (*P*-450) reductase/haemoprotein close to that in the intact liver microsomal fraction. As can be seen, the time course of reaction is biphasic, with 62% of cytochrome *P*-450 being reduced in the fast phase (Fig. 7).

Since, in the biphasic processes presented throughout Figs. 6 and 7, the fraction of cytochrome *P*-450 reacting in the fast phase predominates, the latter was considered representative. Accordingly, the apparent first-order rate constants calculated for the fast phases of the individual reactions are included in Table 3 for comparison with the other kinetic data.

Discussion

Although cytochrome *P*-450 is known to effect hydroperoxide-supported *C*-oxidation of a wide variety of compounds, the ability of the haemoprotein to mediate peroxidatic attack on nitrogen

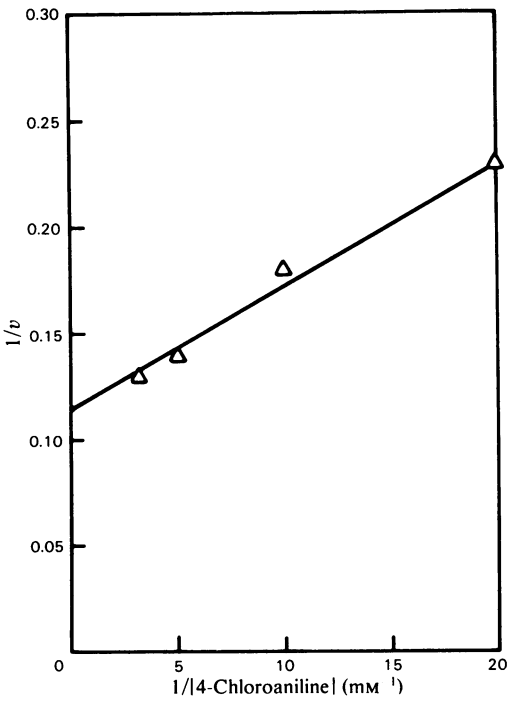

Fig. 5. *Initial-velocity pattern for the NADPH-driven microsomal N-oxidation of 4-chloroaniline with amine substrate as the variable*

Microsomal suspensions, as described in the Materials and methods section, containing NADPH-generating system and 4-chloroaniline as indicated were incubated for 3 min at 37°C. The initial velocities of *N*-oxidation (v) are expressed in nmol of *N*-(4-chlorophenyl)hydroxylamine formed/min per nmol of cytochrome *P*-450. The experimental points are means of three determinations.

centres has been questioned (O'Brien, 1978). The present study confirms that cytochrome *P*-450 can serve as a catalyst in the cumene hydroperoxide-dependent *N*-oxidation of 4-chloroaniline. Analogous to the NADPH/O_2-driven process, product dissociation is likely to limit the overall rate of cytochrome *P*-450 cycling in the peroxidatic pathway too (Table 3). However, there appear to exist differences between both types of reactions as regards the oxy complexes involved. Thus, the 437 nm-absorbing oxy species is stable enough to be observable under steady-state conditions (Fig. 2*b*). The time-dependent transformation of the initial spectral change might result from gradual cytochrome *P*-450 destruction (Akhrem *et al.*, 1977). Probably, the 437 nm-absorbing oxy complex is not directly involved in substrate oxidation, since it decays at a rate far below that of product release (Table 3). Indeed, the spectral intermediate observed after mixing cytochrome *P*-450 with cumene hydroperoxide has been proposed to be generated in a non-productive side reaction (Blake & Coon, 1981*a*).

On the other hand, the oxy complex formed in the presence of O_2, NADPH and amine substrate is very short-lived and, therefore, can be detected only under rapid-reaction conditions (Fig. 3). The compound readily changes to the 452 nm-absorbing intermediate (Fig. 2*a*), which is likely to represent a product adduct of cytochrome *P*-450 arising from oxidative attack on the vulnerable nitrogen atom of the arylamine (Mansuy *et al.*, 1978).

The discrepancy in the positions of the Soret maxima in the absolute spectra of the individual oxy complexes (Fig. 3) might be taken as an indication of subtle structural differences. Most interestingly, modification, by treatment with *p*-chloromercuri-

Table 3. *Apparent rate constants for the cumene hydroperoxide- and NADPH-supported oxidative metabolism of 4-chloroaniline by rabbit liver microsomal cytochrome P-450*

The data are taken from the fast-phase portions of the kinetic tracings shown in Figs. 6 and 7, the double-reciprocal plots presented in Figs. 4 and 5, or are calculated from the sources indicated.

Hydroperoxide-dependent system		NADPH/O_2-dependent system	
Parameter	$k_{app.}$ (s^{-1})	Parameter	$k_{app.}$ (s^{-1})
Binding of 4-chloroaniline	3.30	Binding of 4-chloroaniline	3.85
Formation of the 440 nm-absorbing oxy complex	0.53	Transfer of the first electron	0.32
Formation of the 437 nm-absorbing oxy complex	4.62	Oxygen association	880*–1000†
Decay of the 437 nm-absorbing oxy complex	0.009	Transfer of the second electron	≧0.32‡
Decomposition of intermediate to release *N*-oxy product	0.15	Decomposition of intermediate to release *N*-oxy product	0.14

* Calculated from the data of Rösen & Stier (1973).
† Calculated from the data of Guengerich *et al.* (1976).
‡ Calculated by the method of Noshiro *et al.* (1981).

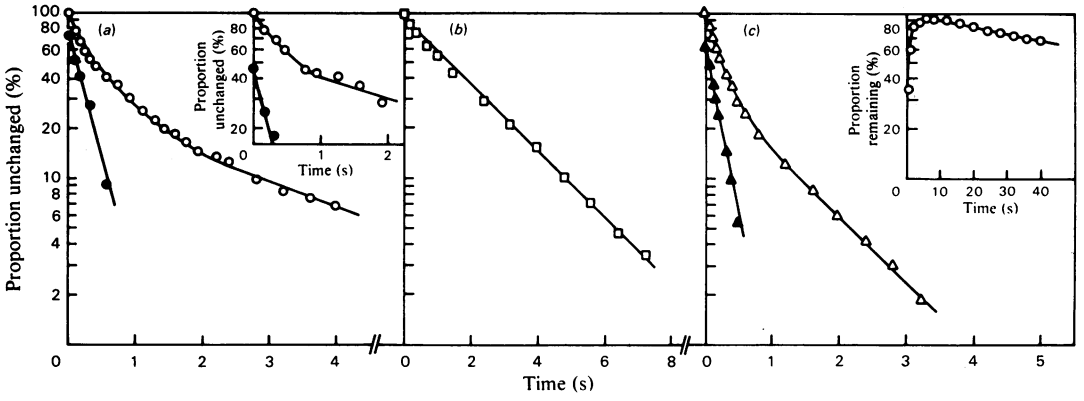

Fig. 6. *Time course of interaction of 4-chloroaniline and/or cumene hydroperoxide with cytochrome P-450*
For experimental details of the stopped-flow technique see the Materials and methods section. (*a*) Binding to cytochrome *P*-450 of 4-chloroaniline, as monitored by the absorbance change at 424nm; the final amine concentrations applied were 1mM (inset) and 10mM respectively. (*b*) Interaction with cytochrome *P*-450 of cumene hydroperoxide, as monitored by the absorbance change at 440nm. (*c*) Interaction with cytochrome *P*-450 of cumene hydroperoxide in the presence of 10mM-4-chloroaniline, as monitored by the absorbance change at 437nm. The inset of the Figure shows the kinetics of decay of the 437nm-absorbing oxy species. The filled symbols in (*a*) and (*c*) refer to the corrected values for the fast-phase portions of the kinetics.

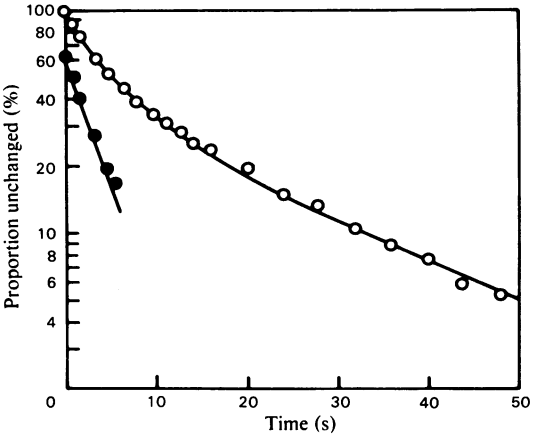

Fig. 7. *Time course of reduction of cytochrome P-450 by NADPH*
The rate of cytochrome *P*-450 reduction by NADPH was determined by monitoring the formation of the CO adduct of the ferrous pigment in the presence of 1mM-4-chloroaniline. For details see the Materials and methods section. The filled symbols represent the corrected values for the fast-phase portion of the kinetics.

benzoate or deoxycholate, of the thiolate ligand in the cytochrome *P*-450 molecule renders the haemoprotein a more powerful peroxygenase (Table 1), but strongly impairs its mono-oxygenating capacity (Hlavica, 1982). Whereas, under mixed-function conditions, the reactivity towards hydroxylatable substrates of the oxygenating species is stimulated through development of various resonance forms via electron abstraction from the intact thiolate group (White & Coon, 1980), such a function appears not to be obligatory when peroxide substitutes for NADPH and O_2.

Serious questions as to the commonness of the oxygenating mechanism operative in the hydroperoxide- and NADPH-supported systems respectively are moreover raised by considering the marked change in product distribution: whereas *N*-(4-chlorophenyl)hydroxylamine, slowly autoxidizing to the corresponding nitrosoarene, is the only product observed with NADPH and O_2, 1-chloro-4-nitrobenzene is the major reaction product generated, along with marginal amounts of the nitroso analogue, when hydroperoxide serves as an oxidant. Such observations contrast with the generally accepted view that organic peroxide-sustained reactions, such as NADPH/O_2-dependent ones, proceed via an oxenoid species generated through heterolytic cleavage of the oxygen donor (O'Brien, 1978). It is therefore proposed that the peroxide-dependent transformation of 4-chloroaniline to the nitro derivative is mediated via sequential one-electron oxidations triggered by homolytic cleavage of the oxidant (Blake & Coon, 1981*b*). Such a mechanism is likely to give rise to a number of free-radical intermediates (Yamazaki & Piette, 1963; Bartsch & Hecker, 1971; Griffin, 1980; Josephy *et al.*, 1982). *N*-(4-Chlorophenyl)hydroxylamine and the corres-

ponding nitrosoarene are believed to represent stable intermediates. Support in favour of such a concept comes from the following observations. The low steady-state level of 1-chloro-4-nitrosobenzene attained with cumene hydroperoxide suggests rapid further oxidation of the compound. Addition of exogenous hydroxylamine or the nitroso analogue to the basic assay medium results in almost quantitative conversion into the nitro derivative. There is a close correlation between rate of disposal of the 1-chloro-4-nitrosobenzene added and the overall rate of peroxidatic *N*-oxidation of 4-chloroaniline (cf. Fig. 1 and Table 3).

Although one-electron oxidation of hydroxylamines to nitroxides has been observed to occur also in the presence of the physiological electron donor NADPH (Stier *et al.*, 1972), such a mechanism is obviously not appropriate to effect further oxidation of these radicals to nitro products. In agreement with this, conversion, in microsomal suspensions containing NADPH, of *N*-hydroxyphentermine into the nitro analogue has been conclusively shown to depend on the presence of endogenously generated H_2O_2 (Sum & Cho, 1979).

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